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1. Clinica Chimica Acta, 1976 Jul 1, 70(1):103-112
2. Trans All-India Inst Ment Health, 1969, Vol 9, pp. 35-38.
3. Neurology, 1968 Apr, 18(4):397-402
4. Path Biol (Paris), 1963 Jun-Jul, Vol. 11, pp. 729-741
5. Clinical chemistry, 1989 Jun, 35(6): 972-974
6. Cancer, 2001 Aug 15, 92(4): 856-862
7. Revue Neurologique, 1992, 148(6-7): 417-422
8. Cancer Research:
   1990 Oct 1, 50(19): 6364-6370
   1987 Jul 15, 47(14):3766-3770
9. Cancer Bull, 1981, 33(6):250-254
10. Acta Nurochirurgica, 1971, 25(1):57-68
11. Neurology, 1968 Apr, 18(4):397-402
12. Int J of Cancer, 1996 Aug 22, 69(4):350-353
13. Clin Chem, 1997 Jan, 43(1):85-91
14. Calcif Tissue Int, 1997 Sep, 61(3):183-188
15. J Natl Cancer Inst, 1998 Jul 1, 90(13):1000-1008
16. Clin Cancer Research, 1999 Dec, 5(12): 3914-3919
17. Br J Haematol, 2000 Dec, 111(4):1118-1121
18. Thyroid, 1998 Aug, 8(8):637-641

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EXPRESSION OF BONE SIALOPROTEIN IN PRIMARY HUMAN BREAST CANCER IS ASSOCIATED WITH POOR SURVIVAL

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We have recently demonstrated that bone sialoprotein (BSP), a bone-matrix protein involved in hydroxyapatite crystal formation, is ectopically expressed in human breast cancers. We explored a possible association between expression of BSP in primary breast cancer and patients' survival. We analyzed BSP expression in 454 breast-cancer patients by immunohistochemistry on archival paraffin-embedded material using an anti-BSP polyclonal antibody. BSP expression was correlated to survival, tumor size, axillary lymph-node status and first site of distant metastasis. Of the breast cancers analyzed, 89% expressed detectable amounts of BSP. We found a statistical association between expression of BSP and poor prognosis as indicated by survival curves analyzed using the log rank and the Gehan methods. BSP expression was significantly higher in breast-cancer patients with axillary lymph-node involvement. Interestingly, survival of patients with positive lymph nodes but BSP-negative tumors was significantly higher than that of patients with no lymph-node involvement but BSP-positive cancers. The frequency of bone metastases was higher in the group of patients with BSP-positive tumors (22%) than in the group with BSP-negative cancers (7%). There was a significant increase in the incidence of lung metastases in patients whose tumors were negative for BSP. Our data show that bone sialoprotein expression in breast cancer is associated with poor prognosis. BSP detection also appears to be a valuable marker with which to identify, among the lymph-node-negative patients, those who have high risk of disease progression.

Quantificative and qualitative modifications of gene expression are common features of cell transformation, tumor invasion and metastasis. These changes are associated with the acquisition of specific phenotypes that allow cancer cells to successfully proliferate and accomplish each step of the metastatic cascade. Generally, the biological importance of specific genes, up- or down-regulated in malignant cells, is related to a specific step of tumor progression. Recently, we found that bone sialoprotein (BSP) was ectopically expressed in human breast-cancer cells (Bellahcène *et al.*, 1994). This phosphoglycoprotein is involved in various aspects of bone physiology, such as the regulation of mineralization during bone resorption and formation (Fisher and Termine, 1985). BSP participates in the initiation of hydroxyapatite deposition and in the interaction between bone cells and the mineralized matrix (Chen *et al.*, 1991; Gehron Robey, 1989). Expression of BSP was thought to be restricted to mineralizing connective tissue with the exception of decidual cells and trophoblasts (Bianco *et al.*, 1989). In our previous study, we demonstrated that 87% of breast carcinomas studied showed a significant increase in BSP expression compared to normal breast tissue. In order to further determine the putative importance of this bone phosphoprotein in breast-cancer progression, we examined the impact of BSP expression in primary breast cancers on patient survival. The relationship between BSP expression and 3 major prognostic factors (tumor size, presence or absence of metastases in regional lymph nodes and presence or absence of distant metastases) was also evaluated in this study.

PATIENTS AND METHODS

Patients

A total of 454 women with breast cancer, surgically treated at the National Tumor Institute in Milan, were considered in this study with no exclusions (consecutive patients). All patients were followed up for at least 20 years or until death. Patients with clinically negative lymph nodes had radical or modified radical mastectomy and no further treatment. Patients with clinically involved regional lymph nodes had radical mastectomy or extended radical mastectomy if the primary tumor was larger than 2 cm in diameter and was localized in the inner mammary quadrants. In patients with histologically positive lymph nodes, surgery was combined with subsequent radiotherapy to the supraclavicular and internal mammary lymph nodes. No chemotherapy was administered prior to the time of relapse. Paraffin sections of placental membranes containing trophoblast tissue were used as positive controls.

Immunohistochemistry

BSP was detected by immunoperoxidase using LF83 rabbit polyclonal antibody kindly provided by Dr. L.W. Fisher (Bone Research Branch, National Institute of Dental Research, NIH). LF83 was raised against a synthetic peptide of human bone sialoprotein (residues 277–294) (Mintz *et al.*, 1993). LF83 has been characterized for immunohistochemical application and its specificity controlled by Western blotting. Immunoperoxidase staining was performed using the ABC Vectastain Elite kit (Vector, Burlingame, CA) according to the supplier's directions. Briefly, tissue sections were deparaffinized in xylene and hydrated in PBS (10 mM sodium phosphate, 0.9% NaCl, pH 7.5). The blocking of endogenous peroxidase was performed with 0.3% H_2O_2 in methanol and the non-specific serum-binding sites were blocked with normal goat serum (1:20). Anti-BSP LF83, at a dilution of 1:1,000 in PBS, was applied and incubated for 2 hr at room temperature. Then the tissue sections were incubated with biotinylated goat anti-rabbit antibody (1:200) followed by exposure to pre-formed streptavidin-biotinylated horseradish peroxidase complex. Peroxidase was revealed by the 3,3'-diaminobenzidine tetrahydrochloride reaction. Finally, sections were counterstained with hematoxylin, dehydrated and mounted. Control experiments included omission of the first antibody and pre-incubation of LF83 anti-serum with a 100 molar excess of the corresponding peptide prior to its use in the immunoperoxidase assay. However, in a few representative specimens, several slides were stained at different times to assess the staining reproducibility.

Evaluation of immunohistochemical staining and statistical analysis

The criteria used in assessing the immunoperoxidase staining of the breast tumors were as follows: the intensity of staining was graded on a scale of 0 to 3, where 0 = no staining of tumor cells, 1+ = weakly positive staining, 2+ = moderately positive staining and 3+ = strongly positive staining of tumor cells. Since many tumors showed some heterogeneous staining, the intensity of tumor staining was then assessed according to the staining of the most positive tumor cells when

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their estimated percentage represented at least 30% of the total area of positive tumor cells. For most of the cases, only one slide was stained. Each tumor was assessed independently by 2 observers who had no knowledge of patients' data. The main endpoint of this study was the evaluation of overall survival of the patients from the date of surgery. The disease-free survival was considered unreliable because of the type of follow-up adopted at the time. The survival rates were calculated by means of the actuarial life-table. The log-rank and Gehan methods were used to statistically analyze the differences between the survival curves. The Chi-square test was used to study possible associations between BSP expression and several parameters of the tumors as well as the site of the first distant metastasis.

RESULTS

Expression of BSP was evaluated in 454 paraffin-embedded surgical specimens of human breast carcinomas. Placental tissue was used as a positive control, since it has been shown that trophoblastic cells express BSP (Bianco *et al.*, 1991). We found that both syncytial trophoblasts and cytotrophoblasts were stained with LF-83 antibody. Out of the 454 lesions examined, 408 (89%) exhibited detectable amounts of BSP. The immunoreactivity was essentially localized to the cytoplasm and the cell surface of breast-cancer cells. Both the pattern and the intensity of staining were reproducible. Surrounding stromal tissue was consistently negative. Normal mammary tissue, when present, always exhibited low or no

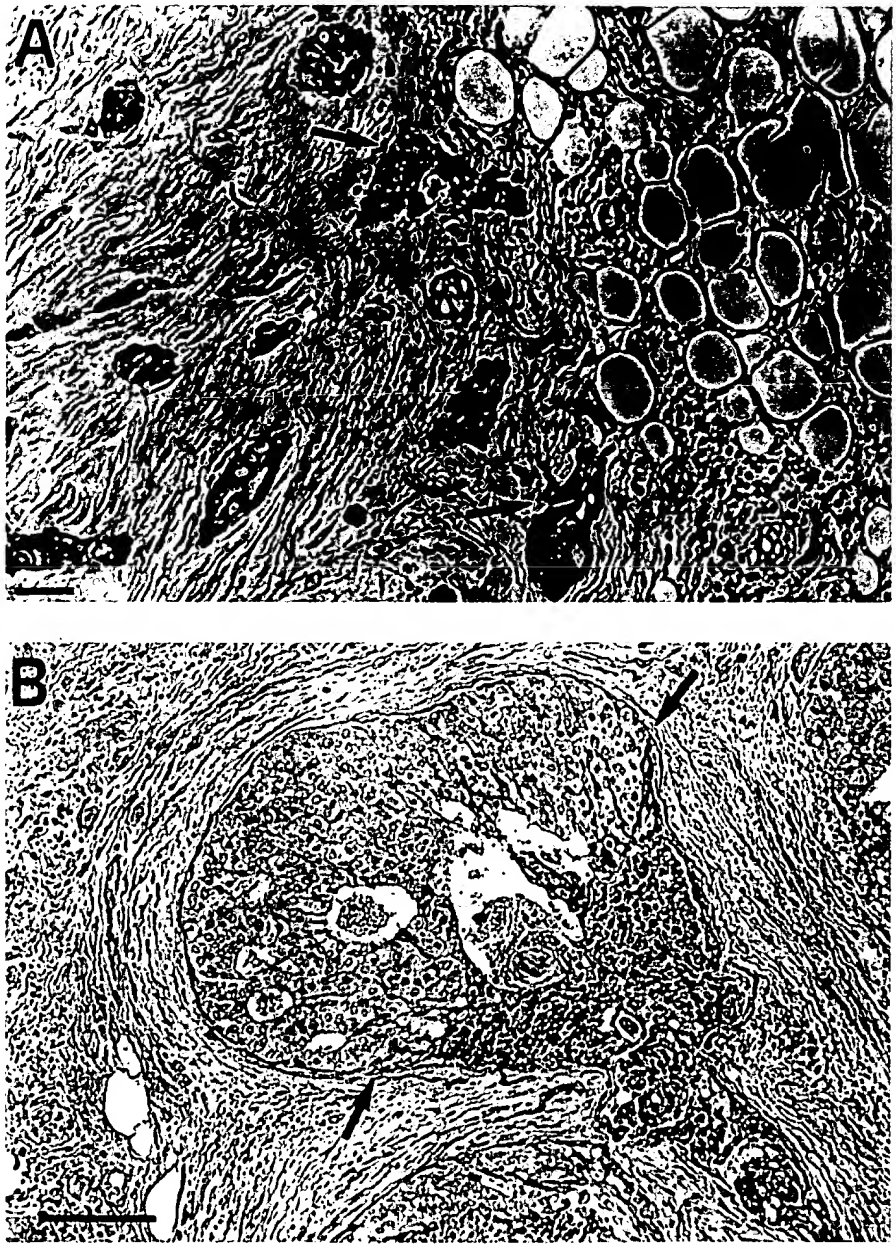

FIGURE 1 – Immunostaining for bone sialoprotein in breast cancer. Paraffin-embedded tissue sections were immunostained with a polyclonal antibody (LF83) and counterstained with hematoxylin as described in the text. *(a)* Infiltrating ductal breast-carcinoma cells invading the surrounding fat tissue (arrows), staining score 3+. *(b)* Infiltrating ductal carcinoma progressing to invasive carcinoma (arrows). The invading component shows an increased BSP expression evaluated as 2+. Scale bars = 100 μm.

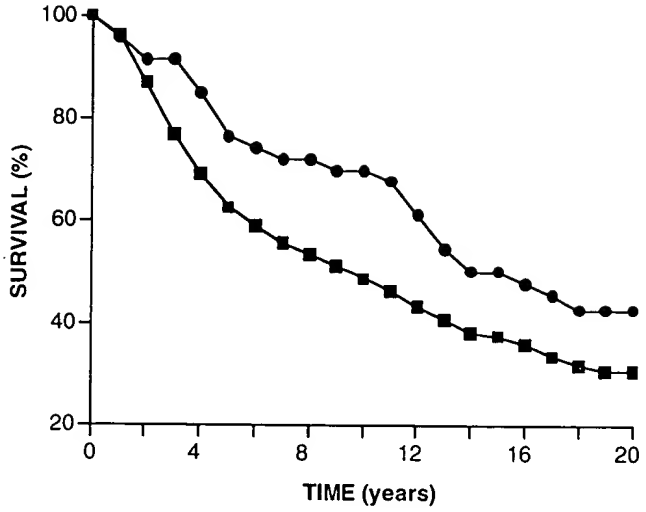

FIGURE 2 – Survival curves according to BSP expression in human breast cancers. ■ = BSP-positive; ● = BSP-negative.

TABLE I – BSP EXPRESSION CORRELATES WITH TUMOR SIZE AND THE AXILLARY LYMPH-NODE STATUS

Prognostic factor	BSP staining		*p*
	Negative	Positive	
Negative lymph nodes	26/42 (62)[1]	167/394 (42)	0.01
Tumor size > 2 cm	20/36 (55)	136/370 (37)	0.02

[1]Number of cases/total; percentage in parentheses.

reactivity to anti-BSP antibody. Positive staining was abolished by omission of the primary antibody and by pre-incubation of LF83 anti-serum with a 100 molar excess with the corresponding synthetic peptide (data not shown). The intensity and pattern of staining were reproducible. In 60% of the cancer lesions examined, staining of the cancer cells was homogeneous, with more than 90% of the cells observed exhibiting the same degree of immunoreactivity. Figure 1*a* shows a representative example of ductal breast-carcinoma cells invading the surrounding fat tissue and expressing high levels of BSP evaluated as 3+. Figure 1*b* illustrates an *in situ* ductal carcinoma with a part of the lesion progressing to an invasive carcinoma. While the cells from the *in situ* lesion are weakly positive for BSP, it appears clearly that expression of BSP is increased in the invasive component of the lesion.

Analysis of the survival data according to BSP expression in the primary breast lesions indicates that it is associated with a poor prognosis. Survival of patients with BSP-negative tumors was better than that of patients with cancers expressing detectable amounts of BSP (Fig. 2). However, no differences in survival were observed according to the different levels of BSP expression, although there was a tendency towards a longer survival for patients whose primary lesion expressed lower levels of BSP (data not shown). Statistical analysis of the survival curves indicated a borderline significance with the log rank test ($p = 0.08$) whereas the Gehan test, which considers principally the first part of the curves, reached significance with $p = 0.04$, indicating that BSP expression is associated with early relapses of the disease. The biggest difference in survival was observed after 10 years from the time of diagnosis. After this period, 70% of patients with BSP-negative tumors were still alive, while only 49% of those whose cancer expressed detectable levels of BSP had survived.

The survival data were also evaluated in subgroups of patients divided according to the 2 most important prognostic

TABLE II – RELATIONSHIP OF BREAST-TUMOR POSITIVITY FOR BSP AND SITES OF FIRST DISTANT METASTASIS

Site of first metastasis	BSP staining		*p*
	Negative (n = 15)	Positive (n = 178)	
Local recurrences	1 (7)[1]	22 (12)	0.72
Regional nodes	2 (13)	6 (3)	0.28
Lung	4 (27)	7 (4)	0.004
Bone	1 (7)	39 (22)	0.22
Liver	0 (0)	8 (5)	0.81
Multiple sites	7 (46)	96 (54)	0.54

[1]Number of cases; percentage in parentheses.

factors in breast-carcinoma staging, which are nodal status and tumor size. Patients with positive lymph nodes but BSP-negative tumors had a better survival than node-negative patients whose tumors expressed BSP ($p = 0.005$, according to the log rank test). There was a statistically significant association between BSP expression in the primary tumor and the presence of metastatic cells in the axillary lymph nodes ($p = 0.01$) (Table I). Tumors larger than 2 cm in diameter were more often negative for BSP than small tumors ($p = 0.02$) (Table I).

We also examined the relationship between BSP expression and the site of first distant metastasis or local recurrence (Table II). Breast cancers expressing BSP metastasized more often to the skeleton (22%) than those which did not express BSP (7%). An intriguing finding was a significant correlation between BSP expression and low frequency of the lung as the first site of dissemination ($p = 0.004$). There were no significant correlations between expression of BSP and local recurrences, regional nodes, liver or multiple-site metastases.

DISCUSSION

In this study, we have examined the prognostic value of BSP in a large retrospective series of 454 breast-cancer patients followed up for at least 20 years. We found that 89% of the patients expressed detectable levels of BSP. Expression of BSP in breast cancer was significantly associated with poor prognosis. As expected, there was a significant association between BSP expression in the primary tumor and axillary lymph-node involvement. Patients with positive lymph nodes but BSP-negative tumors had a better survival than lymph-node-negative patients with BSP-positive breast cancers. This observation is interesting since it indicates that BSP detection could help identify, within a group of breast-cancer patients with negative axillary lymph nodes, those who are at high risk of disease progression. Because of early detection of breast cancer, the axillary lymph-node status has less prognostic significance. Also, it is a major challenge to identify reliable predictors of the metastatic potential of lymph-node-negative breast cancer. If BSP production by primary tumors provides a means of identifying those at risk of progression, it will facilitate the selection of the lymph-node-negative patients who are most likely to benefit from specific adjuvant treatments. BSP is a secreted protein that can be detected in the serum (Chenu *et al.*, 1994). If the expression of BSP by breast-cancer cells results in a significant increase in serum BSP levels, then RIA or ELISA assays in the serum of breast-cancer patients could be used as quantificative assays for clinical evaluation.

We also found that patients whose tumors express BSP were more likely to develop colonies in the bone as first site of dissemination. This observation is in agreement with our preliminary study of 39 patients showing that detection of BSP in primary malignant breast lesions could significantly predict the risk of development of bone metastases (Bellahcène *et al.*, 1996*b*). Bone is the target of predilection for circulating metastatic breast-cancer cells. Bone metastases are respon-

sible for significant morbidity due to pain, pathological fractures, hypercalcemia and bone-marrow replacement (Body, 1992; Hortobagyi, 1991; Nielsen *et al.*, 1991). The pathophysiology of bone metastases is poorly understood. Breast-cancer cells express other bone-matrix proteins such as osteonectin and osteopontin (Bellahcène and Castronovo, 1995). Like BSP, these proteins are involved primarily in bone-tissue mineralization (Gehron Robey, 1989). It is unlikely that the preferred homing of circulating malignant breast-cancer cells to bone and the ectopic expression of bone-matrix proteins by breast-cancer cells are fortuitous. However, a direct relationship between these 2 features remains to be demonstrated. The recent reports that breast-cancer cells do indeed attach to BSP *in vitro* could shed light on the way in which BSP participates in the osteotropism of cancer cells (van der Pluijm *et al.*, 1993, 1995). Through its RGD domain, BSP mediates osteoclast binding to the bone matrix prior to its resorption (Ross *et al.*, 1993). In the same way, this domain could play an important role in mediating the attachment of circulating breast-cancer cells. Recently, we showed that BSP is indeed expressed at the mRNA and protein levels in human breast-cancer cell lines (Bellahcène *et al.*, 1996*a*). Interestingly, BSP was expressed at the cell surface of estrogen-receptor-positive, but not estrogen-receptor-negative, breast-cancer cells. These data are in agreement with our hypothesis that cell-surface BSP could facilitate interactions of metastatic breast cancer cells with bone tissue. We found a significant correlation between BSP expression and lung metastases as the first site of metastatic dissemination. In fact, patients whose tumors were BSP-positive were less likely to develop lung metastases. The observation that BSP-positive tumors metastasize preferentially to bone rather than to lung suggests that the expression of this bone-matrix protein by breast-cancer cells plays a role in their selective affinity for the skeleton. Ongoing prospective studies should confirm the value of BSP detection in primary breast cancers to permit evaluation of overall prognosis and risk for specific metastatic sites.

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REFERENCES

BELLAHCÈNE, A., ANTOINE, N., CLAUSSE, N., TAGLIABUE, E., FISHER, L., JARES, P. and CASTRONOVO, V., Detection of bone sialoprotein in human breast tissue and cell lines at both protein and messenger ribonucleic acid levels. *Lab Invest.*, (1996*a*). (In press).

BELLAHCÈNE, A. and CASTRONOVO, V., Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer. *Amer. J. Pathol.*, **146**, 1–6 (1995).

BELLAHCÈNE, A., KROLL, M., LIEBENS, F. and CASTRONOVO, V., Bone sialoprotein detection in human breast cancer cells: a potential predictor of bone metastases. *J. Bone Min. Res.*, **11**, 665–670 (1996*b*).

BELLAHCÈNE, A., MERVILLE, M.-P. and CASTRONOVO, V., Expression of bone sialoprotein, a bone matrix protein, in human breast cancer. *Cancer Res.*, **54**, 2823–2826 (1994).

BIANCO, P., FISHER, L. and YOUNG, M., Expression of bone sialoprotein in human developing bone as revealed by immunostaining and *in situ* hybridization. *J. Bone Min. Res.*, **4** (Suppl.), S246 (1989).

BIANCO, P., FISHER, L.W., YOUNG, M.F., TERMINE, J.D. and ROBEY, P.G., Expression of bone sialoprotein (BSP) in developing human tissues. *Calcif. Tissue Int.*, **49**, 421–426 (1991).

BODY, J., Metastatic bone disease: clinical and therapeutic aspects. *Bone*, **13**, S57–S62 (1992).

CHEN, J., SHAPIRO, S.S., WRANA, J.L., REIMERS, S., HEERSCHE, J.N.M. and SODEK, J., Localization of bone sialprotein (BSP) expression to sites of mineralized tissue formation in fetal rat tissues by *in situ* hybridization. *Matrix*, **11**, 133–143 (1991).

CHENU, C., IBARAKI, K., GEHRON, R.P., DELMAS, P.D. and YOUNG, M.F., Immunolocalization of osteopontin, osteocalcin, and dentin sialoprotein during dental root formation and early cementogenesis in the rat. *J. Bone Min. Res.*, **9**, 833–841 (1994).

FISHER, L.W. and TERMINE, J.D., Noncollagenous proteins influencing the local mechanisms of calcification. *Clin. Orthopaed. rel. Res.*, **200**, 362–385 (1985).

GEHRON ROBEY, P., The biochemistry of bone. *Endocrinol. Metabol. Clin. N. Amer.*, **18**, 859–902 (1989).

HORTOBAGYI, G., Bone metastases in breast cancer patients. *Semin. Oncol.*, **18**, 11–15 (1991).

MINTZ, K.P., GRZESIK, W.J., MIDURA, R.J., GEHRON ROBEY, P., TERMINE, J.D. and FISHER, L.W., Purification and fragmentation of nondenaturated bone sialoprotein: evidence for a cryptic, RGD-resistant cell attachment domain. *J. Bone Min. Res.*, **8**, 985–995 (1993).

NIELSEN, O., MUNRO, A. and TANNOCK, I., Bone metastases: pathophysiology and management policy. *J. clin. Oncol.*, **9**, 509–524 (1991).

ROSS, F., CHAPPEL, J., ALVAREZ, J., SANDER, D., BUTLER, W., FARASH-CARSON, M., MINTZ, K., GEHRON ROBEY, P., TEITELBAUM, S. and CHERESH, D., Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin $\alpha_v\beta_3$ potentiate bone resorption. *J. biol. Chem.*, **268**, 9901–9907 (1993).

VAN DER PLUIJM, G., IVANOV, B., ROBEY, F., GEHRON ROBEY, P., PAPAPOULOS, S. and LÖWIK, C., Bone sialoprotein peptides are potent inhibitors of breast cancer cell adhesion to bone *in vitro*. *J. Bone Min. Res.*, **10**, Suppl. 1, S177 (1995).

VAN DER PLUIJM, G., KERR, J., LOWIK, C. and GEHRON ROBEY, P., β_1 and β_3 integrin subunits are involved in adhesion of breast cancer cells to extracellular bone matrix. *J. Bone Min. Res.*, **8**, Suppl. 1, S136 (1993).